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EFFECT OF AUXIN AND CYTOKININ FOR *INVITRO* REGENERATION OF *PRUNUS DOMESTICA* L. VAR. PIONEER EXPLANTS: AN ECONOMICALLY IMPORTANT FRUIT TREE

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ABSTRACT

This study was conducted to know the effect of Auxin and Cytokinin type and its concentration for invitro propagation of Prunus domestica L.var. Pioneer explants. Pioneer explants were sterilized by using different concentration of sodium hypochlorite and mercuric chloride, and then cultured on Murashige and Skoog media supplemented with different concentration of BAP or KIN alone and in combination with IBA. The shoots were transferred to half strength MS medium supplemented with varying concentrations of IBA or IAA for root growth and development. Explants disinfected with sodium hypochlorite (2%) for 15 minutes and mercuric chloride (0.1 %) for 7 minutes has highest significant survival value. The highest growth and development rate of shoots was obtained on media with BAP and poor multiplication was achieved on media with KIN. From different combinations of KIN or BAP with IBA, the highest shoot induction was observed on MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L IBA with an average number of 3.18 ± 0.58 shoots per explants and 3.36 ± 0.29 cm average shoot length. The highest rooting was observed on 1.0 mg/L IBA with an average number of 4.05 ± 1.2 roots per shoot and 3.66 ± 0.1 cm average root length.

KEYWORDS: Pioneer, In vitro regeneration, Explants & MS media

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Original Article

INTRODUCTION

Temperate tree fruit are belonging to Rosaceae plant family, which include plum, sour cherry, sweet cherry, apricot, almond, peach, apple, nectarine, and pear (Hummer and Janick 2009). These fruits are grown from tropical to subtropical region of the world (Gemu and Handoro, 2006; Melke and Fetene, 2014).

Plums, and their dried form (prunes), have laxative, anticancer, anti-hyperglycemic, anti hyperlipidemic, antihypertensive, anti-osteoporosis, and hepatoprotective activities because of their lower fat, carbohydrates (sorbitol, glucose, fructose and sucrose), amino acid, organic acids (Malic acid, citric, tartaric, benzoic and boric acid), vitamins (A, B_1, B_2, C, and K), minerals (potassium, calcium, magnesium, zinc, copper, manganese, selenium and boron), dietary fibers and polyphenolic compounds (Bofung *et al.,* 2002; Folta, 2009; Jabeen and Aslam, 2011; Prajapati *et al.,* 2012; Topp *et al.,* 2012 and Nisar *et al.,* 2015).

Temperate fruit breeding via conventional method is a difficult and expensive process due to self incompatibility, polyploidy, long breeding cycles, and lengthy field trial procedures (Canli and Tian, 2008). Most

of these fruit trees are propagated asexually such as grafting and cuttings, however these processes are seasonal dependent and labor intensive ((Botu *et al.,* 2002; Beckman and Lang, 2003; Botu and Botu, 2007; Hartmann and Neumuller, 2009 and Topp *et al.,* 2012, Pipinis *et al.,* 2012, Esen *et al.,* 2007 and Hjeltnes and Nornes, 2007, Ghayyad *et al.,* 2010, Druart, 1992 and Webster, 1995).

Micro- propagation through tissue cultures will overcome these drawbacks, as well as providing virus- disease- free plants so that preventing disease spreading among orchards (Rosati *et al.,* 1980; Nedelcheva *et al.*, 1985; Mante *et al.,* 1989; Bassi and Cossio, 1991; Mante *et al.,* 1991; Druart, 1992; Nowak and Miczynski, 1996 and 1997; Silva *et al*., 2003; Tian *et al.,* 2007; Ruzic and Vujovic, 2007; Zs *et al.,* 2008; Zou, 2010; Ostadsharif *et al.,* 2014 and Choudhary *et al.,* 2015). Therefore, the developed protocols here would be suitable bio techniques for fruit tree industry.

There is no universal medium for *in vitro* culture because plant species and cultivars are genetically specific with regard to different components of the medium. Murashige and skoog medium (1962) proved to be the most suitable medium for successful explant development of stone fruit (almond, apricot and peach) (Zaied, 1997). One of the most important aspects of successful micropropagation were determination of an effective sterilization protocol, optimal types and proper concentrations of Auxin and Cytokinin as medium constituents (Mante *et al.,* 1989 and 1991; Tian *et al.,* 2007; Thorpe *et al.*, 2008; Mansseri-Lamrioui *et al.*, 2011 and Nazary and Aghaye, 2012). Therefore, this study was conducted to investigate the effect of Auxin and Cytokinin for in vitro regeneration of *Prunus domestica* L. Var. Pioneer explants

MATERIALS AND METHODS

Stock Solution and Media Preparation

MS media (Murashige and Skoog, 1962) were prepared by dissolving the appropriate amount of macro and micro nutrient, and organic supplements. Plant growth regulators (BAP, KIN, IBA and IAA) stock solutions were prepared using the proportion of 1 mg: 1 ml and stored in a refrigerator at 4°C for further use. The MS culture media were prepared from its respective stock solutions using 3% sucrose, different concentration of plant growth regulators and agar (7 g/L) for shoot initiation and multiplication. The plant growth regulator BAP or KIN (0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) and in combination with IBA (0. 0.5, and 1.0 mg/l) were added separately to the media to study the effect on shoot proliferation. 55 ml of the medium were dispensed in each culture jars and autoclaved at 121°C for 25 min., after adjusting the pH to 5.8 with 1 N NaOH or 1 N HCl.

Effect of Sterilization Treatments during Culture Establishment

This experiment was made to investigate effect of sterilizing agents for effective sterilization of explants used for in vitro regeneration. The explants were prepared by taking growing nodal segments by removing extra leaf sheaths. Explants were rinsed thoroughly in soap water then washed by running tap water for 15 to 30 min. to remove soil and other superficial contamination. Then the nodal segments were rinsed in sterile-distilled water for 30 min. The explants were rinsed for 20 min. with sterile cold anti-oxidant solution (150 mg/L citric acid and 100 mg/L ascorbic acid) to avoid a browning problem of the tissue in the culture.

The explants were soaked for 30 min. in 70% ethanol under aseptic conditions in a laminar air-flow cabinet. To develop a successful sterilization protocol the following sterilization treatments were used. The prepared explants were immersed in different concentrations of NaOCl (1%, 2%, and 3% (v/v)) and/or mercuric chloride (MC) at concentrations of

0.05 %, 0.1% and 0.2 % (w/v) for different exposure times (**Table 1**) with a few drops of Tween-20. Each treatment consisted of three jars, each jar containing four axillary bud as explants source. After disinfection treatments, the explants were thoroughly rinsed for 4-5 times to remove all traces of the disinfectants and attached sterilizing agent using sterile double distilled water. The sterilized bud segments were kept in fresh sterile double distilled water for a maximum of 30 second until final trimming and culturing them on a basal MS medium (Murashige and Skoog, 1962).

The culture jars with cultured explants were securely sealed with Parafilm™, labeled and then the cultures were transferred to the growth room with 16 hours photoperiod (8 hours dark) and 2700 lux light intensity at 25 ± 2 ^{O}C. Observations were recorded regularly during 30 days to identify the non-growing cultures, infected cultures and healthy cultures. Then the surviving explants were taken as a source for the plant material used for the following experiments.

Effects of Auxin and Cytokinin Alone and with Combination for Shoot Initiation and Multiplication

This experiment was designed to study the effect of different concentrations of BAP or KIN (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/L) alone and in combination with 0.1 mg/L IBA on shootlet development of the explants of *Prunus domestica* L. Var. Pioneer. To obtain high proliferation of shoots, the explants were sub cultured three times on the best medium to obtain stock materials to be used for the following experiments. One month after the third subculture (after three months from the first subculture, or starting the experiment), the number of shootlets/explants and shoot let length (cm) were recorded.

Statistical data Analysis

Completely randomized designs with three replications were made and a maximum care was taken to minimize any variation in the laboratory conditions among treatments for each of the experimental materials. Statistical data analysis was done by using Excel spreadsheets and SPSS version 16.0. To detect the significance of differences among treatments at $p \leq 0.05$, analysis of variance (ANOVA) was used. Means of different treatments were compared by using Duncan Multiple Range Test at a 5% confidence interval.

RESULTS AND DISCUSTIONS

Sterilization of *Prunus domestica* L. var. Pioneer explants for culture establishment

One of the challenges faced in *in vitro* propagation of plant was the microbial contamination at the initiation and multiplication stages. The microbes competed with plant tissue culture for nutrients; hence they increased explants' mortality, reduced shoot proliferation and rooting, and contributed to tissue necrosis and growth abnormality (Kane, 2003). Most likely it is impossible to prevent contamination of the in vitro-grown plants unless preventative measures are taken. In a majority of commercial and scientific plant tissue culture laboratories, the losses due to contamination were between 3 and 15% for every in vitro subculture (Leifert *et al.,* 1994). This issue resulted in economic losses because of waste of time, effort and materials (Webster *et al.,* 2003). Especially, the elimination of microorganism in woody plant material was problematic (Niedz and Bausher, 2002).

During micropropagation the methods of surface sterilization of explants were depending on, plant species, surface contaminant levels, growth environment, age and part of the plant used for micro propagation (Sathyanarayana and Varghese, 2007). Therefore, this experiment was conducted to study the effectiveness of sodium hypochlorite and/or

mercuric chloride for surface sterilization of *Prunus domestica L. var.* Pioneer explants for micro propagation. After three to four days of bud transfer to sterile MS medium, the growth of microorganisms (bacteria and/or fungi) was observed around the base of the explants. This problem can be caused by insufficient aseptic techniques during working, incomplete surface sterilization of the explants and microorganism available in the explants (Constantine, 1986; Buckley and Reed, 1994). The responses of explants to various types and concentrations of sterilization agents were different. The analysis of variance (ANOVA) and the level of statistical significance ($p \leq 0.05$) for the contamination, survival and damage of explants are presented in **Table 1**.

The data as shown in **Table 1** revealed that as the concentration of sodium hypochlorite increased from 1% to 3%, contamination was decreased, and the same was true when the concentration of $HgCl_2$ increased from 0.05% to 0.2% for almost all levels of exposure time. There were non-significant levels of low contamination and minimum explant death when explants were disinfected with 1% NaOCl for 20 min. in combination with 0.2 % $HgCl_2$ for 5 min. and 2% NaOCl for 15 min. in combination with 0.1 % $HgCl_2$ for 5 min. The highest significant survival value (97%) was recorded when explants were disinfected with 2% NaOCl for 15 minutes and 0.1 % $HgCl_2$ for 7 min (**Figure 1**). It is known that, in order to reduce the rate of explants mortality during surface sterilization, the sterilizing agent concentration should be reduced as the exposure time increased and vice versa to minimize the phototoxic activity of the sterilizing agents (Sathyanarayana and Varghese, 2007).

According to Moutia and Dookum (1999), none of the surface-sterilizing agents were effective for total elimination of microorganism using sodium hypochlorite alone or mercuric chloride alone. In this experiment, the positive effect of the combination of methods may be due to a sufficient synergistic effect of $HgCl_2$ and NaOCl on suppression of the survival of microorganism within a short period of time, hence it does not affect the cultured explants. Similarly, other researchers (Sundari *et al.*, 2011, Ranyaphi *et al.* 2012, and Arun and Swamy 2015) used the sodium hypochlorite in combination with mercuric chloride for effective sterilization of different explants. However, there was slight modification of concentration of mercuric chloride and sodium hypochlorite including the exposure time. This change may be due to variation of plant materials taken for sterilization.

Table 1: Establishment of an Effective Sterilization Condition for Invitro Regeneration

Treatments and Exposure Time(Minutes)	Contaminated buds (%)	Survival buds (%)	Damaged buds (%)
1% NaOCl for 30 min	100^{a}	0^{e}	0^{b}
2 % NaOCl for 25 min	94^{a}	5.53^{de}	0^{b}
3% NaOCl for 15 min	74.67^{c}	2.77^{de}	19.43^{a}
0. 05% $HgCl_2$ for 15 min	91^{ab}	8.3^{cd}	0^{b}
0. 1% $HgCl_2$ for 10 min	80.33^{bc}	13^{c}	0^{b}
0.2 % $HgCl_2$ for 7 min	77.67^{c}	0^{e}	22.2^{a}
2 % NaOCl for 15 min and 0.1% $HgCl_2$ for 7 min	3^{d}	97^{a}	2.77^{b}
1 % NaOCl for 20 min and 0.2% $HgCl_2$ for 5 min	2.59^{d}	91^{b}	5.53^{b}

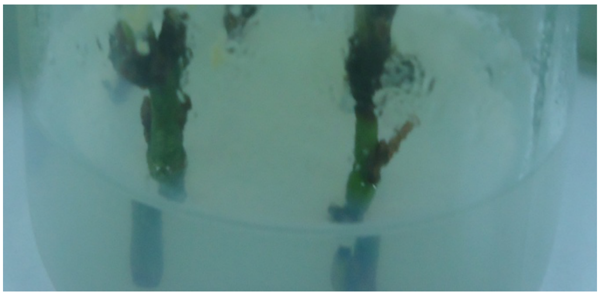

Figure 1: The Best Surviving and Healthy Explants were Obtained During Surface Sterilization of Axillary Buds Using 2 % NaOCl for 15 min and 0.1% $HgCl_2$ for 7 min

Shoot induction and multiplication

Different concentrations of plant growth regulators such as BAP and KIN alone or in combination with IBA were evaluated for the maximum production of multiple shoots. After 4 weeks of culture, shoot number and shoot length was observed and recorded. The cultures were sub cultured at an interval of 3 weeks for three times for multiple shoot proliferation.

The results indicated that the medium containing 0.5 mg/L BAP or 1 mg/L BAP in combination with 0.1 IBA showed significantly similar positive response for shoot induction and shoot multiplication **(Figure 2)**. Hence, the use of the lower concentration of BAP is recommended because it is more economical. The results are in line with those of prior researchers (Silva *et al.*, 2003, Emarah, 2008, Mansseri-Lamrioui *et al*., 2011, Ostadsharif *et al*., 2014, Ruzic and Vujovic, 2008 and Zou 2010). In comparison to KIN, BAP has high physiological capacity to break apical dominance and promote shoot proliferation at low concentrations (Silva *et al.*, 2003; Pruski *et al.*, 2005; Tian *et al.*, 2007; Zou, 2010 and Edriss *et al.*, 2014). MS initiation medium supplemented with different concentrations of KIN, and combinations of KIN with 0.1mg/L IBA, induced a lower mean number of shoots compared to MS medium prepared with different concentrations of BAP, and combinations of BAP with 0.1mg/L IBA, as shown in **Table 2.**

In order to obtain the maximum number of shootlets in the cultured plants, shoots were repeatedly subcultured three times in similar media composition with the same time intervals. The subculturing was done on MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L IBA. The highest shoot induction was observed on MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L IBA, with an average number of 3.18 ± 0.58 shoots per explants, and 3.36 ± 0.29 cm average shoot length. The results of this study revealed that subculturing in the prescribed sequence of stages used here has no significant effect on the multiplication rate of the shoots. This was in agreement with a study by Vujovic *et al.* (2012).

Table 1: Shoot Number and Shoot Length Produced at Different Concentration of (0-3mg/L) BAP in Combination with 0.1 mg/L IBA

BAP (mg/L) +0.1mg/L IBA	Number of Shoots	Shoot Length (cm)
0	1.00 ± 0.00^{a}	1.66 ± 0.50^{a}
0.5	3.18 ± 0.58^{b}	3.36 ± 0.29^{b}
1	2.59 ± 0.44^{bc}	2.50 ± 0.50^{a}
2	2.67 ± 0.58^{bc}	2.93 ± 0.153^{ab}
3	1.00 ± 0.58^{a}	1.43 ± 0.29a

Means annotated with the same superscript letters in the same column are not significantly different at the 5% probability level.

Figure 2: Shoots Produced in MS Medium Supplemented with 0.5 mg/L BAP in Combination with 0.1 mg/L IBA

Root initiation

The shoots produced in vitro during shoot proliferation were transferred to half strength MS media supplemented with different levels of IBA and IAA in mg/L (0.5, 1.0, 1.5, 2.0 and 3.0) for root induction and development of root systems. In this experiment half strength MS media was used. The mineral concentrations of the culture medium contribute in the process of regulating hormonal balance for root initiation. It is well known that half strength MS media reduces callus formation, and some researchers have proposed the superiority of half strength MS medium for root induction (Zou, 2010, Choudhary *et al.,* 2015, Hossain *et al.*, 2003, and Alam and Barua, 2015). Data, including percentage of rooting, roots/explants and average length of roots (cm) as affected by the type of auxin concentration, are presented in **Table 3** and **Figure 3.** The highest rooting percentage (100%) and significantly highest root number of 4.05 ± 1.2 and root length of 3.66 ± 0.1 cm was obtained in half strength MS media supplemented with IBA (1mg/L). Poor root growth and development was reported in half MS media supplemented with IAA and in the control treatments, without IAA. Half strength MS media supplemented by IBA, with concentrations other than 1mg/L IBA, gave low root production as well. The highest concentrations of IBA proportionally encouraged tissue lignification, leading to considerable decrease in rooting ability. Similar results were previously reported in other temperate fruit species by many researchers (Bandeira *et al.,* 2012, Hossain *et al.*, 2003, Mansseri-Lamrioui *et al.*, 2011, Vujovic *et al.*, 2012 and Alam and Barua, 2015).

The lower response of IAA and higher response of IBA for root induction might be due to rapid phytochemical and enzymatic oxidization by an oxidase. IBA apparently oxidized slowly. IBA can enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA. Thus, keeping cultures in the dark for a short period of time prior to transferring to light conditions can enhance in vitro rooting ability, because photoreceptor

activation in dark is one of the factors, which is involved in plant growth processes (Housman, 2003, Nazary and Aghaye, 2012 and Mansseri-Lamrioui *et al.*, 2011).

Table 3: Root Induction on Half Strength MS Medium Supplemented with Different Concentration of IBA

IBA (mg/L)	Percentage rooting	Number of roots	Root length(cm)
Control	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
0.5	70.66 ± 0.10^c	3.17 ± 1.25^c	2.46 ± 0.3^d
1.0	100 ± 0.00^d	4.05 ± 1.2^d	3.66 ± 0.1^e
1.5	71.67 ± 0.00^c	3.28 ± 0.9^c	2.53 ± 0.5^d
2.0	29.73 ± 0.00^b	1.78 ± 0.2^b	1.9 ± 0.45^c
3.0	29.22 ± 0.00^b	0.5 ± 0.4^a	0.9 ± 0.8^b

*The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

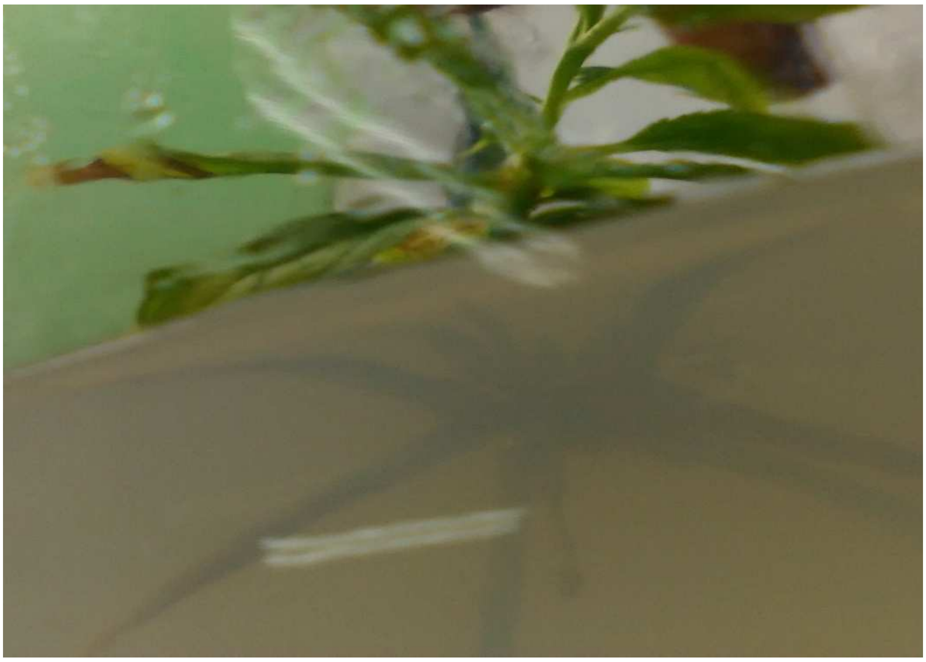

Figure 1: Root Initiation of Pioneer Shootlets on Half Strength MS Media Supplemented with 1mg/L IBA

CONCLUSIONS

In this study the effect of Auxin and Cytokinin was investigated for invitro propagation of plum (Prunus domestica L.var. pioneer) explants. During sterilization protocol establishment the highest significant survival value (97%) were recorded when explants disinfected with 2% sodium hypochlorite for 15 minutes and 0.1 % mercuric chloride for 7 minutes. The growth and development of shoots and roots of this fruit plants were dependent on the combination and concentration of Auxin and Cytokinin. The best shoot response and proliferation was obtained on full strength MS media supplemented with 0.5 mg/L BAP in combination with 0.1 mg/L of IBA. Whereas, the best rooting response was observed on half strength MS media supplemented with 1.0 mg/L IBA. Therefore, the above Auxin and Cytokinin type and concentrations are recommended for in vitro propagation Prunus domestica L. Var. Pioneer explants

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